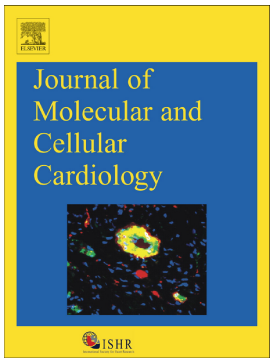

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DKK3 overexpression attenuates cardiac hypertrophy and fibrosis in an angiotensin-perfused animal model by regulating the ADAM17/ACE2 and GSK-3β/β-catenin pathways

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Abstract

Aims: Cardiac pressure and humoral factors induce cardiac hypertrophy and fibrosis, which are characterized by increased stiffness, reduced contractility and altered perfusion. Angiotensin II (AngII) is well known to promote this pathology. Angiotensin-converting enzyme (ACE) 2, which cleaves AngII and forms Ang-(1–7), exerts protective anti-hypertrophy and anti-fibrosis effects. A disintegrin and metalloproteinase 17 (ADAM17), a membrane-bound enzyme reported to cleave ACE2, may participate in the pathological process of AngII perfusion-induced heart damage. However, researchers have not clearly determined whether dickkopf-3 (DKK3) regulates the ADAM17/ACE2 pathway and, if so, whether DKK3-mediated regulation is related to the glycogen synthase kinase-3β (GSK-3β)/β-catenin pathway. In this study, we explored whether DKK3 overexpression ameliorates the development of AngII-induced cardiac fibrosis and hypertrophy through the ADAM17/ACE2 and GSK-3β/β-catenin pathways.

Methods: Mice were injected with a DKK3-overexpressing adenovirus or vehicle and then infused with AngII or saline using subcutaneously implanted mini-pumps for four weeks. Hearts were stained with hematoxylin-eosin, Masson's trichrome and immunohistochemical markers for histology. Primary fibroblasts were treated with the adenovirus and AngII and then examined using western blotting, EdU (5-Ethynyl-2'-deoxyuridine) assays and immunofluorescence. *Additionally, siRNA silencing was performed to study the role of DKK3 and the involved pathways*.

Results: AngII-induced cardiac hypertrophy and interstitial and perivascular fibrosis

were less severe in DKK3-overexpressing mice than in control mice. Moreover, the expression levels of fibrotic genes, such as collagen I and III, and the hypertrophic genes atrial natriuretic peptide (ANP) and beta-myosin heavy chain (β-MHC) were decreased. DKK3 overexpression also exerted a protective effect by inhibiting ADAM17 phosphorylation, thus increasing ACE2 expression and subsequently promoting AngII degradation. Furthermore, this process was mediated by the inhibition of GSK-3β and β-catenin and decreased translocation of β-catenin to the nucleus. *On the other hand, the DKK3 knockdown by siRNA achieved opposite results.*

Conclusion: DKK3 overexpression substantially alleviated AngII infusion-induced cardiac hypertrophy and fibrosis by regulating ADAM17/ACE2 pathway activity and inhibiting the GSK-3β/β-catenin pathway.

Keywords: DKK3, ADAM17/ACE2, GSK-3β/β-catenin, cardiac hypertrophy, fibrosis, angiotensin

Introduction

Cardiac hypertrophy is a maladaptive reaction in response to pressure or volume stress. The main pathological changes in cardiac hypertrophy present as changes with many aspects, including myocardial interstitial cell hypertrophy, myocardial interstitial cell proliferation, and extracellular matrix increase of myocardial cells, thus leading to myocardial remodeling [1]. Fibrosis constitutes a major hypertrophic pathological development in addition to myocardial cell hypertrophy. *In the pressure-overloaded heart, myocardial cells undergo hypertrophy to increase their strength, and the heart size changes slightly at first; however, under persistent high-pressure conditions, the heart enters a decompensatory period and undergoes eccentric hypertrophy. Zhang et al studied pressure overload-induced cardiac hypertrophy in a mouse model using the aortic banding method. They focused on a small molecule called dickkopf-3 (DKK3), which also aroused our interest. DKK3, a secreted protein in the dickkopf family, is a robust inhibitor of the Wnt signaling pathway. The dickkopf gene family comprises four evolutionarily conserved members (DKK 1-4) and dickkopf-like acrosomal protein 1 (DKKL1) or soggy. Multiple studies have reported DKK3 down-regulation in a variety of cancer cell lines and tissues [11, 12]. In the study by Zhang et al, DKK3 was reported to act as a cardioprotective regulator of pressure-induced cardiac hypertrophy via the regulation of ASK1-JNK/p38 signaling. Moreover, neurohumoral factors such as the renin-angiotensin system (RAS), aldosterone, catecholamine and endothelin, are common causes of cardiac hypertrophy. Among these factors, the RAS is the main and*

most influential pathogenic factor. Several studies have indicated that activation of the RAS contributes to cardiac remodeling [5-7]. Angiotensin II (AngII) has a central role in mediating most of the effects of the RAS and is a key trigger of heart hypertrophy and fibrosis [8]. Angiotensin-converting enzyme (ACE) 2 is an enzyme that hydrolyzes AngII into Ang-(1–7) [9]. Ang-(1–7) also has a broad range of effects on cardiovascular tissues, such as improving vascular endothelial function, decreasing matrix metalloproteinase 9 (MMP9) expression and reducing neutrophil and macrophage infiltration [10, 11]. Adisintegrin and metalloproteinase (ADAM) 17, also known as TNFα-converting enzyme (TACE), is a member of the superfamily of Zn-dependent metalloproteases; it is a type I transmembrane protein that plays a key role in regulating the proteolytic release of some cytokines, chemokines, growth factors and their receptors from cellular membranes [12]. ADAM17 was recently shown to cleave ACE2, leading to ACE2 down-regulation [13]. Aside from aortic banding, AngII infusion is another classical method of constructing cardiac hypertrophy mouse models; this method was particularly suitable for the present study due to the direct use of AngII. However, the mechanism by which DKK3 contributes to AngII-induced cardiac hypertrophy remains to be elucidated. Currently, no studies have examined the relationships between DKK3, ADAM17, and ACE2. Therefore, we proposed the hypothesis that DKK3 inhibits AngII-induced cardiac hypertrophy and fibrosis by decreasing ADAM17 activity and increasing ACE2 expression. Because DKK3 regulates the glycogen synthase kinase-3β (GSK-3β)/β-catenin pathway in many cell types and diseases [14-16], we determined whether DKK3 controls

ADAM17/ACE2 via this pathway.

The present study was designed (i) to determine whether DKK3 expression changes in an AngII-perfused animal model, (ii) to determine whether DKK3 overexpression attenuates cardiac hypertrophy and fibrosis, (iii) and if so, to determine whether this protective response is related to the regulation of the ADAM17/ACE2 pathway, (iv) and whether the ADAM17/ACE2 pathway is modulated by the GSK-3β/β-catenin pathway.

Methods

2.1. Primary cultures of cardiac fibroblasts

Primary cardiac fibroblasts (CFs) were prepared from the hearts of 1- to 3-day-old neonatal C57BL/6 mice, as previously described [12]. Briefly, after sacrifice by decapitation, the sternum of the neonatal mice was longitudinally incised to expose the heart. Then, the heart was isolated and cut into pieces in DMEM. The pieces were digested with collagenase type II for 3 h at 37°C. Then, the liquid supernatant was centrifuged, and cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Gibco, California, USA) and penicillin-streptomycin (100 U/ml penicillin, 100 g/ml streptomycin) (Gibco, California, USA) at 37°C with 5% CO_2. Two hours later, the culture medium was replaced to remove all cells except the CFs. The cells were subcultured in subsequent experiments.

2.2. Cell transduction and drug administration

The DKK3-overexpressing adenovirus was purchased from Abm (Cat: 190468A, Canada) and amplified by the Chinese National Human Genome Center, Beijing. CFs

were pretreated with DKK3 overexpression adenovirus or adenovirus vehicle (MOI=100) for 24 h before AngII (1 μg/ml) stimulation and were cultured for an additional 48 h to evaluate the effect of DKK3 overexpression [17]. The CFs were divided into six groups as follows:

Group 1 (OV+AngII): DKK3-overexpressing adenovirus+ Angiotensin II

Group 2 (AD+AngII): adenovirus vehicle+ Angiotensin II

Group 3 (AngII): Angiotensin only

Group 4(OV): DKK3-overexpressing adenovirus

Group 5(AD): adenovirus vehicle

Group 6 (NC): no additions

The DKK3 small interfering experiment (siRNA) transfection experiment and grouping are described in detail in the supplement.

2.3 Animal model

Based on the results of the preliminary in vitro experiment, the AngII group presented similar results as the AD+AngII group, and the results observed in the NC group were similar to the AD group. We removed the AngII and NC groups to conduct our in vivo experiment with fewer animals. Sixty male wild-type C57BL/6 mice (10–12 weeks of age) were chronically infused with saline or AngII at a rate of 1000 ng/kg/min using osmotic mini-pumps (Alzet, Cupertino, CA) for 28 days as described previously [18]. The DKK3-overexpressing adenovirus or vehicle (2×10^9 pfu) [19] was administered by a caudal vein injection 3 days before the operation. *On day 15*, mice were injected again. Mice were divided into four groups randomly and

treated as follows:

Group 1 (OV+AngII): DKK3-overexpressing adenovirus injection + Angiotensin II pumping

Group 2 (AD+AngII): adenovirus vehicle injection + Angiotensin II pumping

Group 3(OV): DKK3-overexpressing adenovirus injection +saline pumping

Group 4(AD): adenovirus vehicle injection +saline pumping

The blood pressure was measured in the tail on day *31 (three days before adenovirus injection plus 28 days' angiotensin II pumping)* before the mice were sacrificed. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document No 55, 2001) and were approved by the Animal Care Committee of Shandong University.

2.4 Quantitative real-time RT-PCR

Total RNA was extracted from the left ventricle of mice or CFs using TRIzol reagent (Invitrogen, CA). A real-time PCR thermocycler (IQ5 real-time PCR cycler; Bio-Rad) was utilized to perform the quantitative real-time PCR. PCR was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 56°C for 10 s. The transcript GAPDH content was quantified as an internal control. The results were calculated using the $2^{-\Delta\Delta CT}$ method.

2.5 Western blot (WB) analyses

Total proteins and nuclear proteins were extracted from CFs or mouse cardiac tissues, and equal amounts of protein were electrophoretically separated and then

transferred to PVDF membranes. The membranes were incubated overnight at 4°C with a primary antibody. The antibodies used in this study were anti-DKK3, anti-collagen I, anti-collagen III, anti-MMP2, anti-MMP9, anti-phosphor-ADAM17 (T735), anti-ACE2 and *anti-TGF-β1* (all purchased from Abcam，Cambridge，UK), anti-ADAM17, anti-GSK-3β, anti-β-catenin, anti-phospho-GSK-3β (Ser9), anti-active-β-catenin (Ser33/37/Thr41), anti-bcl-2, anti-bax, *anti-Smad3, anti-phospho-Smad3(Ser423/425)* and anti-β-actin (all purchased from Cell Signaling Technology, Boston, USA). Anti-rabbit IgG and anti-mouse IgG antibodies (ZSGB-Bio, Peking, China.) were used as secondary antibodies. The membranes were developed using Immobilon Western Detection Reagents (Millipore, Billerica, MA, USA).

2.6 Cell proliferation assay (EdU)

Cell proliferation was determined with an EdU incorporation assay, as previously described [20]. Briefly, CFs were seeded into slides and administered different drugs or adenovirus the day before staining. CFs were supplemented with 1% FBS and 20 μM 5-ethynyl-2'-deoxyuridine (EDU) (RiboBio, Guangzhou, China). Cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Then, 50 μl of 2 mg/ml glycine were added to each well to neutralize the PFA. The cells were exposed to 100 μl of Apollo-Fluor (RiboBio, Guangzhou, China) for 30 min in the dark at room temperature. Cells were stained with the 1× Hoechst 33342 solution for 30 min. Each well was washed twice with PBS, and images were acquired under a florescence microscope. The percentages

of EDU-positive cells were determined, and three independent experiments were performed.

2.7 Immunofluorescence

CFs were briefly washed with cold PBS 3 times and fixed with 4% paraformaldehyde for 30 min. Then, the CFs were washed thrice, blocked in 5% goat serum albumin for 30 min, and permeabilized using Triton X-100. Cells were incubated with a primary rabbit anti-active-β-catenin antibody overnight at 4°C, followed by an incubation with the secondary antibody (ZSGB-Bio, Peking, China). Nuclei were stained with DAPI for 5 min at room temperature, and immunofluorescence was analyzed under a florescence microscope.

2.8 ELISA

The level of DKK3 in mouse plasma was measured using ELISA kits (kit 50247, SinoBiological Inc., Wuhan, China), according to the manufacturer's instructions. The levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in mouse plasma were measured using ELISA kits (eBioscience, San Diego, USA). *The level of Ang-(1-7) in mouse plasma was measured using an ELISA kit (J&L Biological, Shanghai, China).* The experiment was repeated thrice, and the average number was adopted.

2.9 Detection of mice echocardiography and pathological samples

Echocardiographic imaging was performed post-operatively. The following parameters were tested: left ventricular ejection fraction (LVEF), ratio of early to late mitral inflow velocity (E/A), fractional shortening (FS), and the thickness of the left

ventricular diastolic posterior wall (LVPWD). Systolic blood pressure was measured using the tail-cuff method at *3 and 31 d* as previously described [18]. Before the mice were sacrificed, body weights were recorded and their tibias were measured. Next, mice hearts were dissected and weighed to calculate the ratio to the body weight. Indexes were calculated including heart weight (HW)/body weight (BW) and HW/tibial length (TL). Mouse hearts were then photographed to observe their morphology.

2.10 Histopathology and immunohistochemistry

Mouse hearts were dissected and fixed in 4% formalin. Heart sections were embedded in paraffin and sectioned to 5-μm thickness for staining with hematoxylin and eosin (H&E). Extracellular matrix (ECM) deposition was valued by staining with masson's trichrome. Slides were incubated overnight with the following primary antibodies: rabbit anti-ACE2, anti-collagen I and III, anti-CD45, anti-CD68 and anti-IL-6 (all Abcam, Cambridge，UK). The sections were incubated with a goat antibody (ZSGB-BIO, Peking, China) as the secondary antibody. Matrix accumulation was quantified using Image- Pro Plus 6.0.

2.11 Statistical analysis

All data are expressed as the mean±standard deviations (SD). Statistical analysis was performed with either Student's t-test or one-way ANOVA as appropriate. *P* values<0.05 were considered statistically significant.

3 Results

3.1 DKK3 overexpression alleviates AngII-induced cardiac dysfunction and

hypertrophy

A mouse model of cardiac hypertrophy was constructed to determine whether DKK3 overexpression exerted a protective effect on myocardial hypertrophy. Mice were chronically infused with AngII at a rate of 1,000 ng/kg/min using osmotic mini-pumps for 28 days to generate cardiac hypertrophy [18]. In our experiment, mice in the treatment group were injected with the DKK3-overexpressing adenovirus, and other groups were treated as described in the Methods. First, immunoblot and ELISA analyses confirmed that the injection of the DKK3-overexpressing adenovirus elevated the DKK3 level in both the cardiac tissue and serum (Fig. 1a-1,1a-2 and 1c). In vitro, DKK3 adenovirus transduction also elevated DKK3 expression (Fig. 1b-1, 1b-2). *In the siRNA transfection experiment, DKK3 expression was inhibited by about 50% in the DKK3-siRNA+AngII group and DKK3-siRNA group compared to the NC-siRNA group (Fig. S1a).* We found that cardiac hypertrophy was successfully alleviated in the DKK3 overexpression group compared to that in the control group. Representative images of each group are shown in Fig. 1d. The inhibition of cardiac hypertrophy was also evidenced by the decreased ratios of HW/BW and HW/TL. *The value of BW was close to that of TL, and there were no significant differences between them; thus, we showed only HW/TL in Fig. 1e.* The cross-sectional area of cardiomyocytes examined with wheat germ agglutinin (WGA) staining revealed an alleviation of AngII-induced cardiac hypertrophy in the DKK3-overexpressing group *(Fig. 2a and 2b).* RT-PCR showed that the hypertrophic markers atrial natriuretic peptide (ANP) and beta-myosin heavy chain (β-MHC) were *markedly* decreased in

AngII-treated DKK3-overexpressing mice compared to those in AngII-treated adenovirus-null mice *(Fig. 2c)*.

Tissue Doppler imaging was adopted to evaluate the function of mouse hearts treated with different drugs. The echocardiography results indicated that DKK3 elevated the decreased LVEF, E/A ratio and FS in AngII-perfused mice. In contrast, the left ventricular (LV) posterior wall thickness at diastole (LVPWd) decreased in the group that was injected with the DKK3 adenovirus and perfused with AngII (Fig. 2d-2h)

Blood pressure rose in the AngII-perfused groups (OV+AngII: 167.8±21.3/115.9±12.3 mmHg *vs.* AD+AngII: 159.3±19.8/112.3±10.4 mmHg) compared to that in the saline-pumped groups (OV:122.5±15.6/91.3±6.7 mmHg *vs.* AD:119.7±13.5/88.7±5.7 mmHg), but no significant differences were detected between the OV+AngII and AD+AngII groups or the OV and AD groups.

3.2 Overexpression of DKK3 inhibits AngII-induced cardiac fibrosis

To further determine whether DKK3 overexpression inhibits AngII-induced cardiac fibrosis, a histological examination was performed on heart sections. Masson's trichrome staining revealed a significant decrease in interstitial and perivascular fibrosis in DKK3-injected and AngII-infused mice compared with those in vehicle-injected mice (Fig. 3a-3b). Immunohistochemistry staining confirmed that the levels of the fibrotic markers collagen I and collagen III were significantly down-regulated in DKK3-treated mice compared with those in vehicle-treated animals after the AngII infusion (Fig. 3a and 3c). The levels of collagen I and

collagen III were detected by WB, which were similar to those detected by immunohistochemical staining (Fig. 3d-3e). *In the siRNA transfection experiment, inhibition of DKK3 by AngII increased the expression levels of collagen I and collagen III compared to NC-siRNA transfection. Collagen I expression in the DKK3-siRNA+AngII group was even higher than that in the NC-siRNA+AngII group, showing the inhibitory role of DKK3 in collagen synthesis in the heart (Fig. S1b-S1c).*

3.3 DKK3 overexpression inhibits the inflammatory reaction caused by AngII perfusion

Cardiac hypertrophy is a type of inflammatory disease. WB showed that expression of the inflammatory cytokine IL-6 was *decreased* in the DKK3 overexpression group in vitro (Fig. 4a-1 and 4a-2). Based on the results of the in vivo histological analysis, the levels of IL-6, T cells and macrophage markers CD45 and CD68 were reduced in the DKK3 group compared with those in the vehicle group (Fig. 4b1-4b4). Moreover, ELISAs revealed a dramatic decrease in the expression of proinflammatory cytokines, including IL-1β and TNF-α, in DKK3-treated mice compared with that in controls after the AngII infusion (Fig. 4c-4d).

3.4 DKK3 overexpression reduces the expression of matrix metalloproteinases (MMPs) in CFs

Fibroblasts secrete MMPs, which are involved in matrix degradation. The accumulated amount and increasing activity of MMPs represents the severity of organ fibrosis. WB was used to evaluate the content of MMPs in vitro. MMP2 and MMP9 expression was increased in the AD+AngII and AD groups, but decreased in the

OV+AngII group (Fig. 4e-1 and 4e-2).

3.5 DKK3 overexpression inhibits proliferation and promotes apoptosis in CFs

As most collagens are produced by CFs, CF proliferation seems to be a crucial event in cardiac hypertrophy pathophysiology. According to the results of the EdU assays, DKK3 overexpression inhibited CF proliferation in the OV+AngII group (Fig. 5a-1 and 5a-2). Meanwhile, the apoptosis markers bax and bcl-2 were detected by WB and a higher ratio was detected in the OV+AngII group, indicating that apoptosis increased upon DKK3 overexpression (Fig. 5b).

3.6 DKK3 overexpression reduces the level of phosphorylated ADAM17, thus increasing the ACE2 content *and Ang-(1-7) concentration*

Immunoblots were performed using total proteins from CFs to examine the relationships between DKK3, ADAM17 and ACE2. DKK3 inhibited ADAM17 phosphorylation, but the total ADAM17 level exhibited little change (Fig. 5c). Meanwhile, the AngII-induced decrease in ACE2 expression was restored by DKK3 (Fig. 5e). Based on the results obtained from the in vitro assessment, ADAM17 phosphorylation decreased and ACE2 expression increased in the OV+AngII group compared to those in the AD+AngII group (Fig. 5d and 5f). We also performed immunohistochemistry to detect ACE2 expression and observed increased ACE2 expression in the OV+AngII group compared to that in the AD+AngII group (Fig. 5g-1 and 5g-2). *Then, we detected the plasma concentration of Ang-(1-7) in mice and found that plasma Ang-(1-7) concentrations in the OV+AngII group were elevated about two-fold compared to those in the AD+AngII group. There was no significant*

difference in plasma Ang-(1-7) concentration among the other three groups (Fig. s2c). This result demonstrated that overexpression of DKK3 elevated ACE2 expression, which then elevated Ang-(1-7) expression. In the siRNA transfection experiment, DKK3-siRNA transfection increased the level of phosphorylated ADAM17 compared to NC-siRNA transfection with or without AngII; on the other hand, the level of ACE2 decreased (Fig. S1d-1e).

3.7 The anti-hypertrophic and anti-fibrotic effects of DKK3 overexpression are linked to its inhibition of the GSK-3β/β-catenin pathway

We examined the levels of the GSK-3β/β-catenin pathway in vitro to further investigate the mechanism by which DKK3 regulates AngII-induced ECM deposition. DKK3 overexpression reduced the p-GSK-3β and active β-catenin levels (Fig. 6b and 6d). Immunofluorescence staining revealed only a very weak β-catenin signal in the nuclei of CFs in the OV+AngII group, but the signal was *markedly* strengthened in the nuclei of CFs treated with AngII alone (Fig. 6a). Based on the immunoblot analysis, p-GSK-3β was also reduced by DKK3 in vivo (Fig. 6c). *In the siRNA transfection experiment, DKK3 silencing increased the levels of activated β-catenin and phosphorylated GSK-3β in the presence of AngII compared to NC-siRNA transfection (Fig. S1f-S1g).* Thus, DKK3 influenced ADAM17/ACE2 activity to reduce the detrimental effects of AngII, potentially by suppressing GSK-3β phosphorylation and β-catenin accumulation in the nucleus.

3.8 DKK3 overexpression protected against cardiac hypertrophy by regulating the TGF-β1/Smad3 pathway

As is known, the TGF-β1 and Smad pathway promotes collagen generation in many tissues and diseases [21-23]. Therefore, in addition to the ADAM17/ACE2 and GSK-3β/β-catenin pathways, we investigated the TGF-β1/Smad3 pathway. As expected, the levels of TGF-β1 and phosphorylated Smad3 increased in groups treated with AngII but decreased when DKK3 was overexpressed by adenoviruses in vitro; on the contrary, CFs treated with DKK3-siRNA plus AngII exhibited increased expression levels of TGF-β1 and Smad3 (Fig. S1h-S1i, S2a-2b).

Discussion

In this study, low levels of DKK3 expression were observed in the hypertrophic myocardium, and DKK3 overexpression reversed the effects of AngII on cardiac hypertrophy and fibrosis. During cardiac remodeling, the most apparent change is enlargement of the heart, which is due to hypertrophy and fibrosis. Based on our observations of the mouse model of AngII-induced cardiac remodeling, DKK3 overexpression ameliorated cardiac hypertrophy by attenuating cardiomyocyte enlargement and the increase in HW/BW and HW/TL and by decreasing the expression of fetal genes, including ANF and β-MHC (Fig.1e, 2a, 2b and 2c), along with fibrosis, during remodeling by decreasing the collagen synthesis (Fig. 3a and 3d).

Cardiac hypertrophy and fibrosis are induced by many factors, such as mechanical pressure overload or the humoral factors endothelin and AngII. AngII, the most important constituent of the renin-angiotensin-aldosterone system (RAAS), exerts direct effects on myocardial cells and CFs, causing cell growth, hypertrophy,

and extracellular matrix accumulation [24, 25]. Numerous interactive signaling pathways transduce the AngII-induced pro-fibrosis and pro-hypertrophy signals to the nucleus, leading to the reprogramming of gene expression. Methods that transform the excessively detrimental AngII into a protective factor must benefit the injured heart. The ACE2/Ang 1-7/Mas receptor axis is a physiological antagonist of the RAS, where ACE2 cleaves AngII and generates Ang-(1-7) [9]. Ang-(1-7) is a factor that protects the cardiovascular system and has a role in improving vascular endothelial function by decreasing MMP-9 expression and reducing neutrophil and macrophage infiltration [10, 11]. *Guo et al found that Ang-(1-7) attenuates AngII-induced cardiac hypertrophy by stimulating the Sirt3-mediated deacetylation of FoxO3a and SOD2 expression. In our study, we also detected the serum concentration of Ang-(1-7) and found that it was elevated by approximately two fold compared to the vehicle group when DKK3 was overexpressed. Interestingly, overexpression of DKK3 without AngII perfusion did not change the concentration of Ang-(1-7), suggesting that DKK3 overexpression was pathology dependent and harmless (Fig. S2c).* Therefore, we proposed the hypothesis that DKK3 overexpression may increase ACE2 expression and thus affect the cardiac impairment caused by AngII. Immunoblots of cell and tissue lysates were performed to verify this hypothesis. The perfusion of AngII in mice and the addition of AngII to the CFs decreased ACE2 expression, which was restored by DKK3 overexpression (Fig. 5e and 5f). *In contrast, DKK3 silencing resulted in decreased of ACE2 expression (Fig. S1e).* According to the immunohistochemical staining, DKK3 overexpression also increases ACE2

expression in the heart tissue (Fig. 5g-1, 5g-2). We examined the pathway upstream of ACE2 to identify the intracellular mechanism by which DKK3 influenced ACE2. AngII was recently shown to induce ACE2 shedding by promoting ADAM17 activity through a positive feedback mechanism [27]. Therefore, we examined the relationship between DKK3 and ADAM17. In our study, the overexpression of DKK3 in CFs had no effect on the total ADAM17 level, but influenced its phosphorylation state. P-ADAM17 levels decreased in the OV+AngII and OV groups (Fig. 5c and 5d) *and increased in the DKK3-siRNA group (Fig. S1d).*

In addition to the ADAM17/ACE2 pathway, DKK3 also inhibits the GSK-3β/β-catenin signaling pathway. GSK-3β, an anti-hypertrophic kinase, is inactivated when it is phosphorylated following AKT activation [28]. Consequently, GSK-3β is recruited to the Frizzled receptor, thereby inhibiting GSK-3β and reducing its availability for binding to β-catenin. β-catenin is an adhesion-associated protein whose phosphorylation causes its degradation in the cytoplasm. As a consequence, accumulated β-catenin in the cytoplasm is shuttled into the nucleus, which augments the expression of pro-hypertrophic and pro-fibrosis genes [29, 30]. The signaling function of β-catenin is primarily regulated by alterations in its stability. Wnt signaling induces stabilization of cytoplasmic β-catenin [31]. Here, DKK3 overexpression resulted in the inactivating phosphorylation of GSK-3β (Fig. 6b-6c). DKK3 is a secreted protein in the dickkopf family and acts as an inhibitor of Wnt/β-catenin signal depending on the cellular context [32]. Hirschy A et al. reported that mice expressing a non-degradable form of β-catenin developed dilated

cardiomyopathy and died after 5 months of age [33]. In our study, the accumulation of β-catenin in the nucleus was suppressed in the DKK3 overexpression group, thus alleviating AngII-induced hypertrophy (Fig. 6a and 6d). *Meanwhile, opposite results were obtained in the siRNA experiment (Fig. S1f-1g).* In this study, the excess accumulation of ECM components, including collagen I and collagen III, decreased in the DKK3 overexpression group *and increased in the DKK3-siRNA group*, indicating that DKK3 suppressed AngII-induced cardiac fibrosis (Fig. 3a-3e, *S1b-S1c).* Moreover, DKK3 overexpression improved cardiac function, as indicated by the LVEF, LVPWD, E/A and FS values, and *blocked* the changes in cardiac structure in AngII-infused mice (Fig. 2d-2h).

Due to the close relationship between the TGF-β1/Smad3 pathway and collagen synthesis, we examined these two molecules both in the DKK3 overexpression experiment and siRNA silencing experiment. Consistent with previous reports [21-23], increased collagen expression accompanied the increase in TGF-β1 and Smad3 expression. Moreover, DKK3 overexpression reduced this abnormity in TGF-β1 and Smad3 expression caused by AngII, whereas DKK3-siRNA aggravated it (Fig. S1h-S1i, S2a-2b).

The mechanism by which DKK3 decreases the synthesis of ECM became the next focus of this study. Fibroblasts are known to secrete ECM, such as collagen I and III, which contribute to the development of cardiac remodeling and fibrosis. Similarly, MMPs, which are involved in matrix degradation, are also secreted by fibroblasts. The accumulated amount and increasing activity of MMPs indicates the severity of

organ fibrosis [34]. In the present study, MMP2 and MMP9 activity increased in AngII-treated fibroblasts, which was reversed by DKK3 overexpression (Fig. 4e-1 and 4e-2). This finding suggests that the protective role of DKK3 might be partially to regulate the MMP balance.

Inflammatory signaling in cardiomyocytes has recently been found to occur as part of the pathophysiologic process in response to cardiac hypertrophy, fibrosis, and dysfunction in heart disease. IL-1β, TNF-α and IL-6 have been shown to exert a causative effect on the development of cardiac hypertrophy and dysfunction [35-37]. In our study, fibroblasts treated with AngII exhibited increased expression of IL-1β, TNF-α and IL-6, which was suppressed by DKK3 overexpression (from Fig. 4a-1 to 4d). This result was consistent with a report that IL-6 genetic deletion ameliorated angiotensin-II-induced cardiac hypertrophy and fibrosis [38]. In addition to IL-6, macrophages and T cells also participate in the development of cardiomyopathy [39]. An experiment in which macrophages were non-selectively depleted at an early stage reduced the cardiac inflammatory response and improved cardiac function [40]. Immunohistochemistry in our experiment showed a decline in CD45 and CD68 contents when cells were treated with DKK3 compared to those in cells treated with AngII (Fig. 4b-1, 4b-3 and 4b-4).

The apoptosis of cardiomyocytes, which react to different pathogeneses, is an important part of cardiac hypertrophy. Inflammation and oxidative stress are directly related to apoptosis in hypertrophic hearts of diabetic patients and animals [41-43]. We performed EdU staining to evaluate the proliferation of CFs and WB of bcl-2

family proteins to detect apoptosis of *CFs*. Bcl-2 proteins are members of the Bcl family that can prevent cell death and increase cell proliferation and growth. In contrast, bax is a protein that can promote cell death [44]. In this study, the ratio of bax/bcl-2 increased in the DKK3 overexpression group compared to that in the AngII-treated group (Fig. 5b). Meanwhile, the percentage of EdU-positive cells in the DKK3 overexpression group decreased compared to that in the AngII-treated group (Fig. 5a-1 and 5a-2).

Our study has a few limitations. For example, the precise relationship between the ADAM17/ACE2 pathway and the GSK-3β/β-catenin signaling pathway requires further study. Overexpression of components of the GSK-3β/β-catenin pathway should verify whether DKK3 loses its inhibitory effect on ADAM17. Therefore, our future studies will perform this overexpression experiment both in vitro and in vivo. Additionally, the use of transgenic animals and the knockdown of the DKK3 gene would provide better data to explain the effect of DKK3. *Although we performed DKK3-siRNA experiments in the present study to evaluate the effects of DKK3 knockdown, transgenic animal experiments would provide better data. Moreover, using neonatal CFs to mimic adult disease is less accurate, as the phenotype and response of adult CFs to AngII and DKK3 overexpression could be very different. Embryonic stem cell-derived adult CFs may help address this problem and will be considered in our subsequent work.*

In conclusion, the current study indicates that DKK3 overexpression effectively reduces cardiac hypertrophy and fibrosis and improves cardiac function in an

AngII-perfused mouse model. These cardioprotective properties appear to be due to direct influences on the ADAM17/ACE2 pathway that may be achieved via the inhibition of the GSK-3β/β-catenin signaling pathway. Importantly, DKK3 attenuates AngII-induced MMP2 and MMP9 expression and inhibits collagen synthesis in vitro. Furthermore, DKK3 overexpression alleviated the inflammatory reaction and proliferation of CFs caused by AngII. Above all, these results confirmed for the first time that DKK3 exerts cardiac protective effects against cardiac remodeling.

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Conflicts of interest

The authors confirm that there is no conflict of interests.

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Legend

Fig. 1: An elevated DKK3 level alleviates AngII-induced cardiac hypertrophy. (a-b) Immunoblots showing the DKK3 protein level in mouse hearts and CFs. Mouse hearts were removed from treated animals, as described in the Methods section, and proteins were extracted to perform the WB experiment. At least three animals from each group were chosen. Proteins were extracted from CFs after treated with adenovirus or vehicle for 24 h and Ang II for additional 48 h. In subsequent experiments, WBs of CFs were performed as described here, unless stated otherwise. The experiment was repeated three times. (c) The DKK3 level in mouse serum was analyzed using an ELISA kit after the animals were sacrificed at the end of the experiment. (d) Representative image of the heart size. (e) Mouse body weights and tibial lengths

were recorded before the animals were euthanized, and the heart weight was recorded at the moment hearts were excised and washed. The HW/TL ratios was calculated and presented as an average value. $^{*}P<0.01$, $^{\#}P<0.05$. *HW represents heart weight. TL represents tibia length. HT represents heart tissue. CF represents cardiomyocyte fibroblast. OV represents overexpression adenovirus. AD represents adenovirus vehicle. AngII represents angiotensin II. NC represents negative control.*

Fig. 2: DKK3 overexpression alleviates AngII-induced cardiac dysfunction and hypertrophy. Cardiac hypertrophy was reflected by measurements of molecular markers and cardiomyocyte staining, and cardiac function was evaluated using echocardiograms. (a) RT-PCR analysis of ANP and β-MHC levels (two markers of cardiac hypertrophy) in the total RNA extracted from mouse hearts. (b and c) WGA staining of heart sections showing the cross-sectional area of cardiomyocytes. (d) M-mode echocardiograms and pulsed-wave Doppler echocardiograms of the mitral inflow. (e) Early to late mitral flow (E/A). (f) Fractional shortening (FS). (g) Left ventricular ejection fraction (LVEF). (h) Thickness of the left ventricular diastolic posterior wall (LVPWD). Each experiment was repeated three times. $^{\wedge}P<0.05$ compared with the AD+AngII group. $^{\&}P<0.05$ compared with the OV group. *OV represents overexpression adenovirus. AD represents adenovirus vehicle. AngII represents angiotensin II. WGA represents wheat germ agglutinin staining.*

Fig. 3: DKK3 overexpression inhibits AngII-induced cardiac fibrosis. The ECM content was detected by Masson's trichrome staining, immunostaining and immunoblots. (a) Masson's trichrome staining and immunostaining for collagen I and

collagen III in heart sections. Scale bar: 20 μm. (b) Quantitative analysis of myocardial fibrosis. (c) Quantitative analysis of collagen I and collagen III expression. (d) Immunoblot analysis of the collagen I and collagen III proteins in CFs. (e) Quantitative analysis of the levels of the collagen I and collagen III proteins. The experiment was repeated three times. $^{\wedge}P<0.05$ compared with the AD+AngII group. $^{\&}P<0.05$ compared with the OV group. $^{\Delta}P<0.05$ compared with the AngII group. $^{л}P<0.05$ compared with the NC group. *OV represents overexpression adenovirus. AD represents adenovirus vehicle. AngII represents angiotensin II. CF represents cardiomyocyte fibroblast*.

Fig. 4: DKK3 overexpression inhibits the inflammatory reaction and increasing MMPs activity caused by AngII perfusion. The inflammatory reaction was assessed by analyzing the levels of the IL-6 protein in CFs, inflammatory cell infiltration, and cytokine levels in serum. (a) Immunoblot analysis of the IL-6 protein in CFs. (b) Immunostaining for IL-6, CD45 and CD68 in heart sections. The levels of CD45 and CD 68 represented the number of T cells and macrophages that had infiltrated the heart, respectively. (c-e) Quantitative analysis of IL-6, CD45 and CD68 levels. (f-g) ELISA of IL-1β and TNF-α level in mouse serum. (h) Immunoblot analysis of the MMP2 and MMP9 proteins in CFs. Each experiment was repeated three times. $^{\wedge}P<0.05$ compared with the AD+AngII group. $^{\&}P<0.05$ compared with the OV group. $^{\Delta}P<0.05$ compared with the AngII group. $^{л}P<0.05$ compared with the NC group. *OV represents overexpression adenovirus. AD represents adenovirus vehicle. AngII represents angiotensin II. CF represents cardiomyocyte fibroblast*.

Fig. 5: DKK3 overexpression inhibits proliferation and promotes apoptosis in CFs, and its anti-AngII effect was linked to the inhibition of the ADAM17/ACE2 pathway. (a) Laser confocal microscopy of EdU staining showing a decrease in CF proliferation in the OV+AngII group. Nuclei are stained blue with DAPI; red indicates the cells undergoing proliferation. (b) The EdU-positive index is expressed as a percentage of cell counts. (c) Immunoblot analysis of bax and bcl-2 proteins in CFs. The results are displayed as the bax/bcl-2 ratio and show an increase in apoptosis in the OV+Ang II group. (d-g) Immunoblot analysis of the ADAM17 and ACE2 proteins in CFs and heart tissues. ADAM17 protein was inhibited in the OV+AngII group while ACE2 protein increased compared to the AD+AngII group. (h-i) Immunostaining for ACE2 in heart sections and the corresponding quantitative analysis. $^{\wedge}P<0.05$ compared with the AD+AngII group. $^{\&}P<0.05$ compared with the OV group. $^{\Delta}P<0.05$ compared with the AngII group. $^{л}P<0.05$ compared with the NC group. $^{K}P<0.05$ compared with the AD group. $^{\eta}P<0.05$ compared with the NC group. *DKK3-OV represents DKK3 overexpression adenovirus. AD-null represents adenovirus vehicle. AngII represents angiotensin II. CF represents cardiomyocyte fibroblast. HT represents heart tissue. EDU represents 5-Ethynyl-2'-deoxyuridine staining, a kind of cell proliferation assay.*

Fig. 6: The regulation of ADAM17/ACE2 pathway of DKK3 is linked to its inhibition of the GSK-3β/β-catenin pathway. (a) Immunofluorescence staining showing the decreased accumulation of active β-catenin in the nucleus of the OV+AngII group, indicating that the β-catenin pathway was inhibited. (b-d) Immunoblot analysis of the GSK-3β and β-catenin proteins in CFs and heart tissues. The p-β-catenin protein was

detected in nuclear protein extracts, and all other proteins were detected in total protein extracts. GSK-3β and β-catenin proteins were inhibited in the OV+AngII group compared to the AD+AngII group. $^{\wedge}P<0.05$ compared with the AD+AngII group. $^{\Delta}P<0.05$ compared with the AngII group. $^{\pi}P<0.05$ compared with the NC group. *DKK3-OV represents DKK3 overexpression adenovirus. AD-null represents adenovirus vehicle. AngII represents angiotensin II. CF represents cardiomyocyte fibroblast. HT represents heart tissue.*

Fig. 1

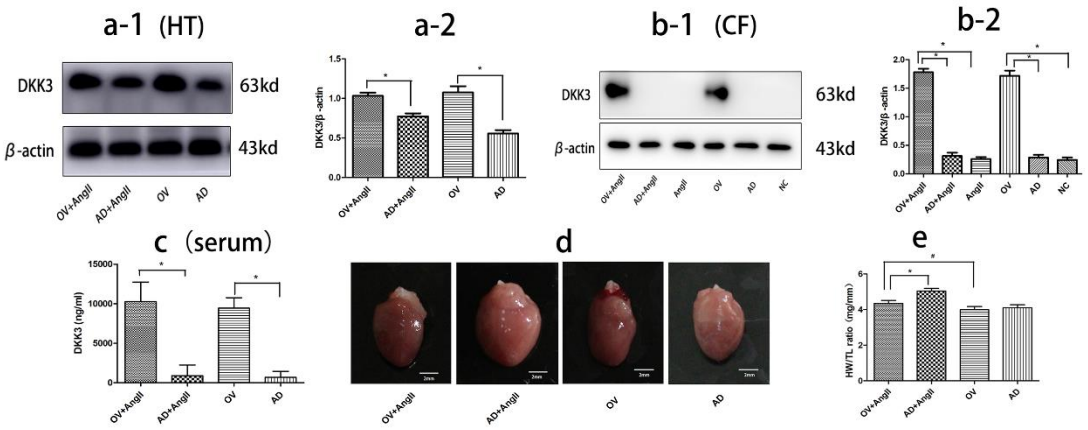


Fig. 2

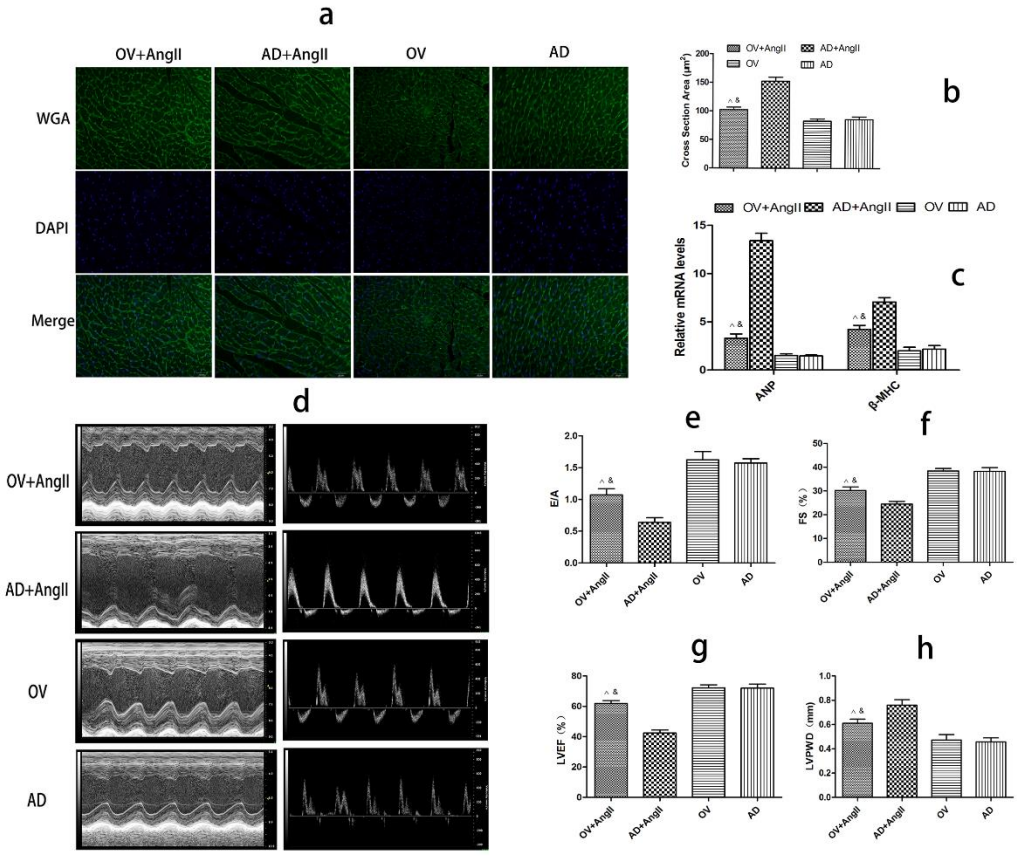


Fig. 3

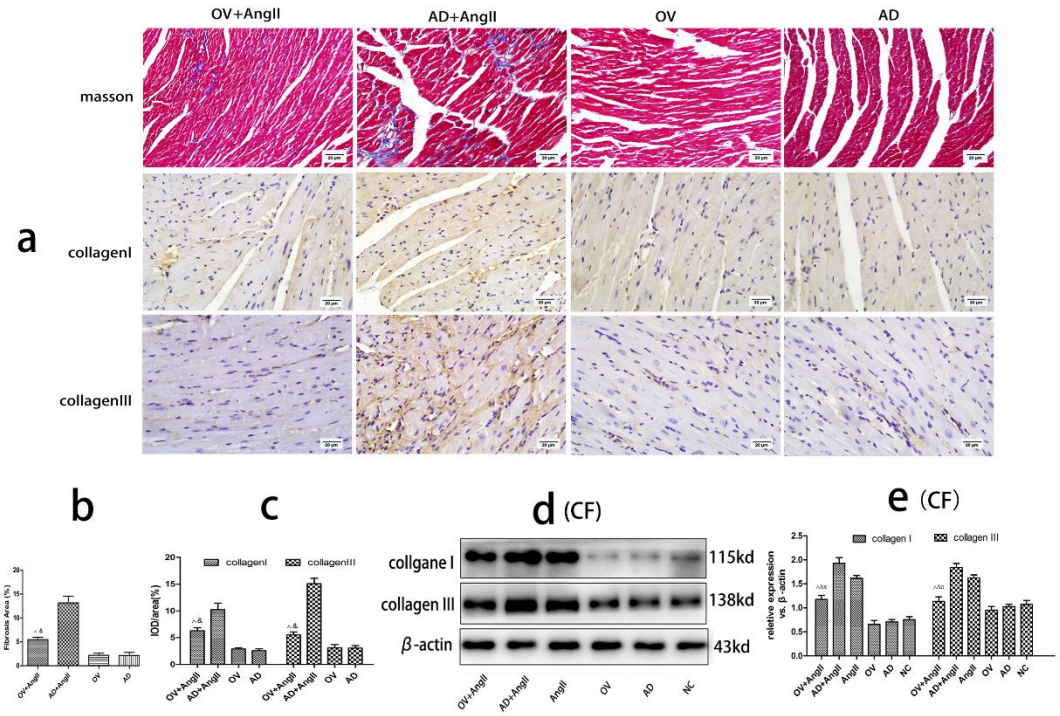


Fig.4

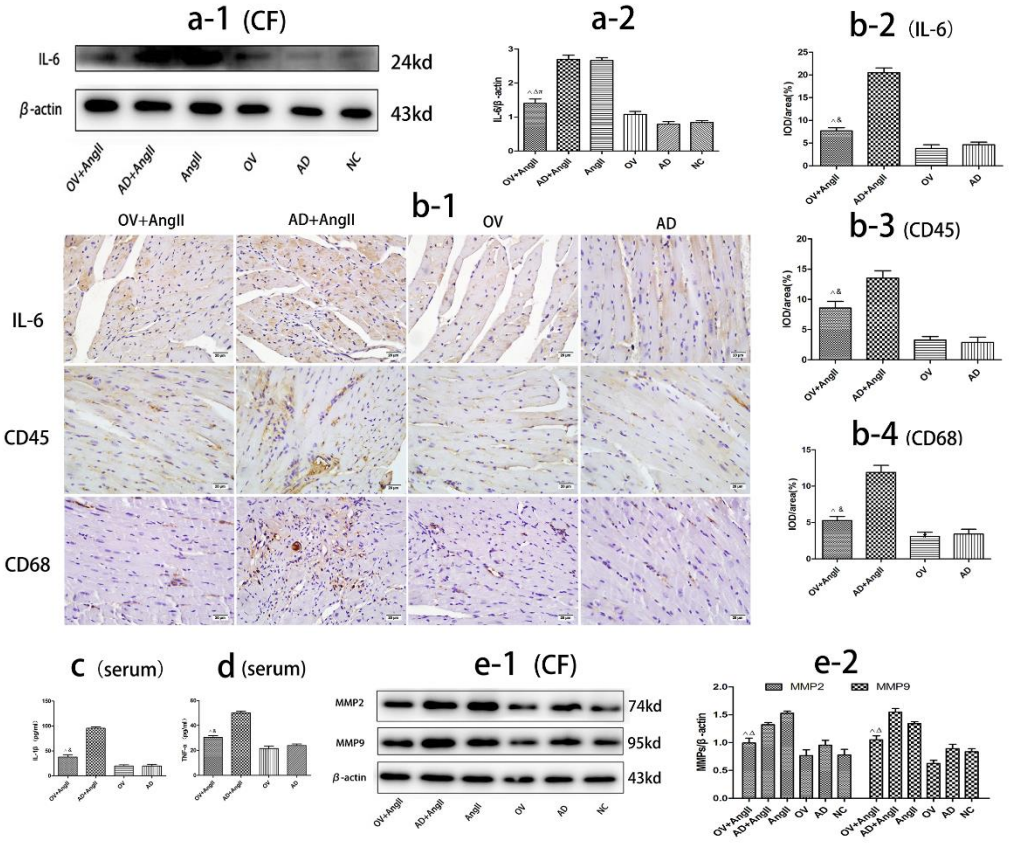


Fig. 5

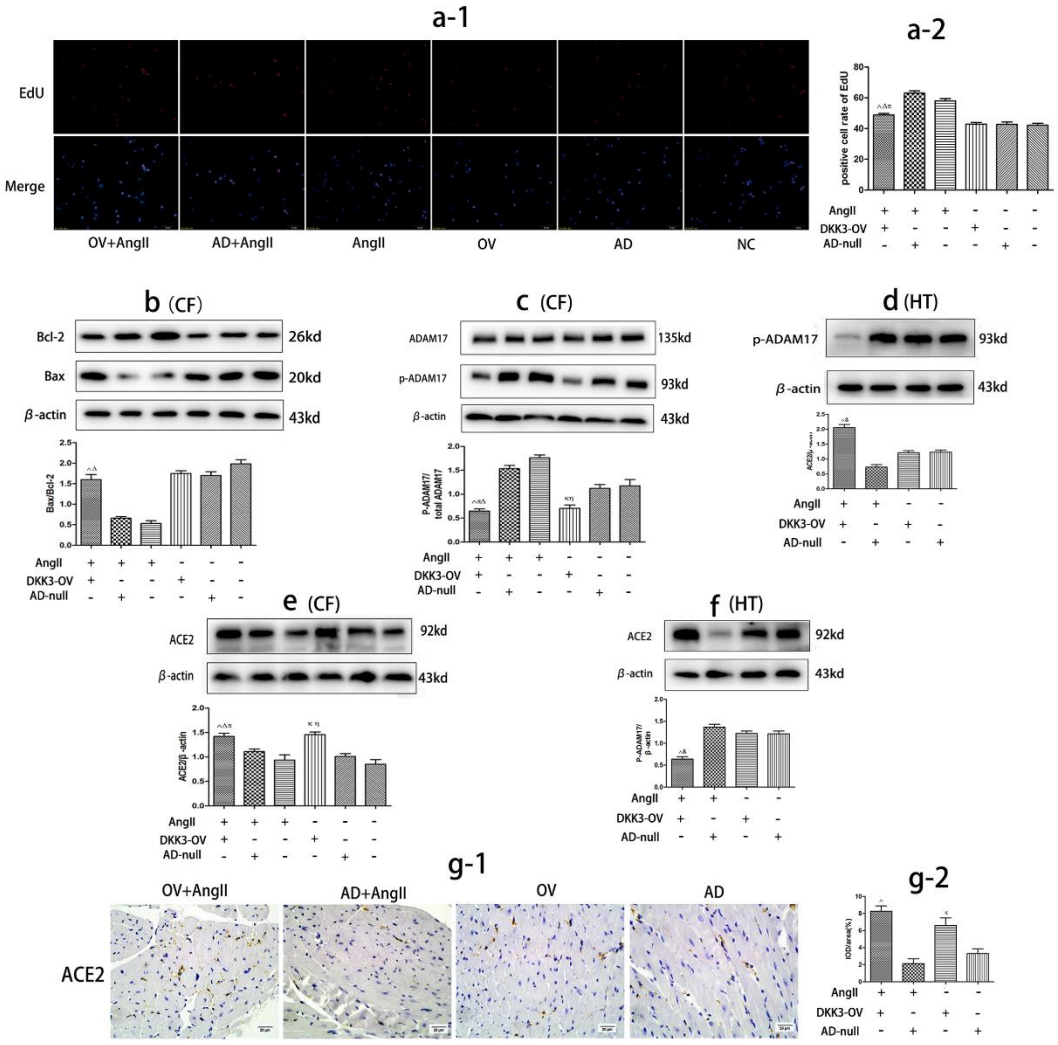
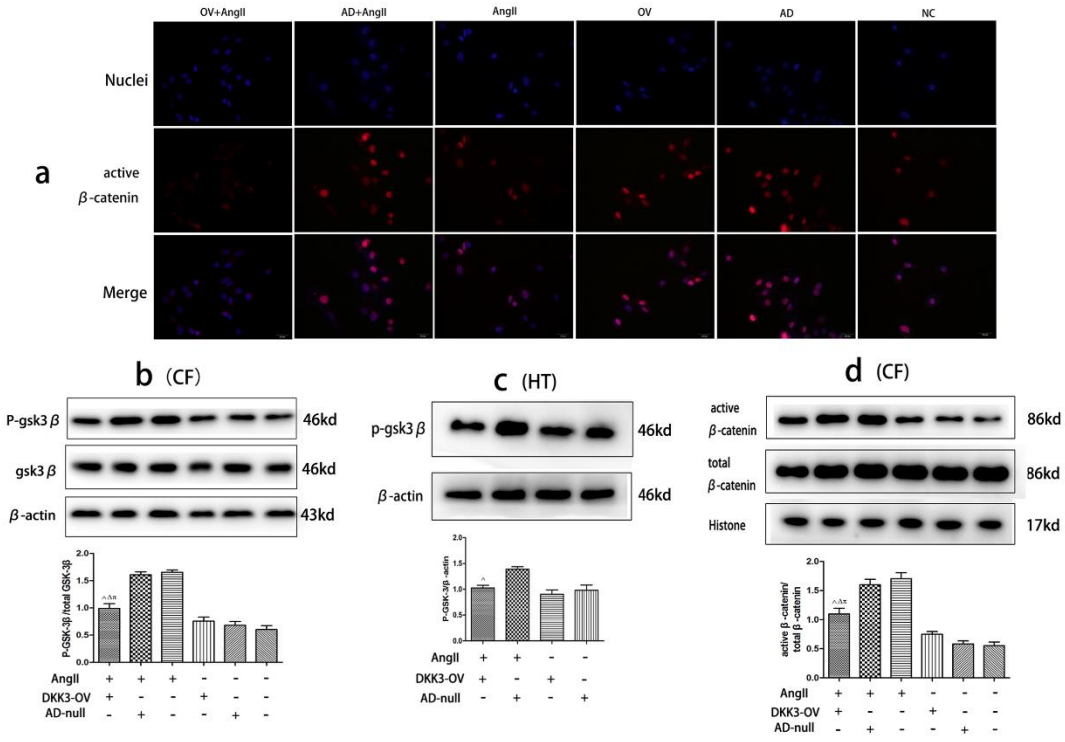


Fig. 6



Highlights

- The current study indicates that DKK3 overexpression effectively reduces cardiac hypertrophy and fibrosis and improves cardiac function in an AngII-perfused mouse model.
- We made some innovations in the mechanism.
- Firstly, we reported the relationship between DKK3 and the ADAM17/ACE2 pathway for the first time. Secondly, we revealed the role of GSK-3β/β-catenin signaling pathway in DKK3 overexpression model.
- Thirdly, we determined the MMPs expression together with inflammatory reaction and proliferation of CFs in hypertrophy heart.
- Above all, these results confirmed for the first time that DKK3 exerts cardiac protective effects against cardiac remodeling by regulating ADAM17/ACE2 pathway activity and inhibiting the GSK-3β/β-catenin pathway.